An Envelope-Specific Glycoprotein from *Escherichia coli* B*

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ABSTRACT: An envelope-specific glycoprotein has been isolated from *Escherichia coli* B. This glycoprotein is phenol soluble and accounts for about 10% of the total cell protein, 35–45% of the total envelope protein, and 50–60% of the partially purified membrane protein. There is about 4% carbohydrate associated with the glycoprotein. *N*-Acetylglucosamine-^{14}C is rapidly incorporated into the glycoprotein, and the amount of incorporation is not appreciably reduced by an excess of amino acids in the growth medium. The glycoprotein is isolated as a phospholipid–glycoprotein complex.

The complex is quantitatively aggregated in the presence of 0.02 M Mg^{2+} or Ca^{2+}. Aggregation is dependent upon the presence of both glycoprotein and phospholipid. Amino acid analysis of the glycoprotein shows that aspartic acid and tyrosine are increased relative to the amino acid composition of isolated envelopes. Acrylamide gel electrophoresis shows that the glycoprotein dissociates in the presence of urea and the multiple-banding pattern is clearest in acidic conditions. Pronase treatment of the glycoprotein (labeled with *N*-acetylglucosamine-^{14}C) produced a series of labeled glycopeptides which were isolated by Sephadex G-25 filtration and high-voltage electrophoresis. At least one glycopeptide contains aspartic acid and glucosamine. The exact linkage was not determined. Inhibitors of protein synthesis such as chloramphenicol or phenethyl alcohol inhibit this glycoprotein synthesis *in vivo*. The envelope-specific glycoprotein of *E. coli* B may have antigenic similarities to beef heart mitochondrial structural protein.

Bacterial membranes from gram-positive organisms have been isolated and well characterized chemically (Salton, 1967a,b). The membranes of *Mycoplasma laidlawii* (Engelman *et al.*, 1967; Terry *et al.*, 1967) and *Micrococcus lysodeikticus* (Butler *et al.*, 1967; Grula *et al.*, 1967) have been disaggregated into small subunits by detergents such as sodium dodecyl sulfate and reaggregated by divalent cations into membrane-like structures when the aggregates were examined by electron microscopy. The membrane-associated enzyme adenosine triphosphatase has been detached from the membranes of *Streptococcus faecalis* and bound again in the presence of Mg^{2+} (Abrams and Baron, 1968). The number of membrane proteins appears to be large. Rottem and Razin (1967) using various *Mycoplasma* strains and Salton (1967a) using *M. lysodeikticus* and *Bacillus subtilis* showed that purified membranes when disaggregated gave very complex patterns in acrylamide gel electrophoresis. Using isolated envelopes (cell wall–cell membrane complex) of the gram-negative organism *Escherichia coli* B we had previously found multiple protein components in this cell fraction (Weinbaum and Markman, 1966) and attempted to characterize enzymatically and chemically these proteins.

It is well known that animal cell membranes contain glycoproteins (Molnar, 1967), but such complex proteins have not been described in bacteria. In this paper we will describe a class of membrane-specific glycoproteins from *E. coli* B and give some physical, chemical, and biochemical characteristics of these interesting compounds.

Experimental Procedure

Preparation of Cell Fractions. Cells of *E. coli* B were grown in glycerol–minimal salts synthetic medium containing 4 g of glycerol, 500 mg of NaCl, 410 mg of $MgSO_4 \cdot 7H_2O$, 1 g of NH_4Cl, 6 g of Na_2HPO_4, and 3 g of KH_2PO_4 per 1000 ml. The bacteria were harvested in late logarithmic phase and washed, and envelopes were prepared as described previously (Weinbaum, 1966). The cell supernatant, after envelope isolation, was opalescent and was fractionated further into a 100,000*g* supernatant and a 100,000*g* pellet. Partially purified membranes were prepared by treating 50 mg of envelopes with lysozyme (200 μg/ml) and EDTA (5 mM) in 5 ml of 5 mM Tris buffer (pH 8.5) and centrifuging the suspension on sucrose according to the method of Kaback and Stadtman (1966).

Isolation of Glycoproteins. Whole cells, envelopes, membranes, cell supernatants, and 100,000*g* supernatants were all subjected to the phenol extraction procedure of Westphal *et al.* (1952) as described by Osborn (1966). Under these conditions the lipopolysaccharide would be extracted into the aqueous phase and the peptidoglycan would be in the residue. Most cell protein would be distributed in the interface, the phenol phase, and the residue. This extraction for glycoprotein is not

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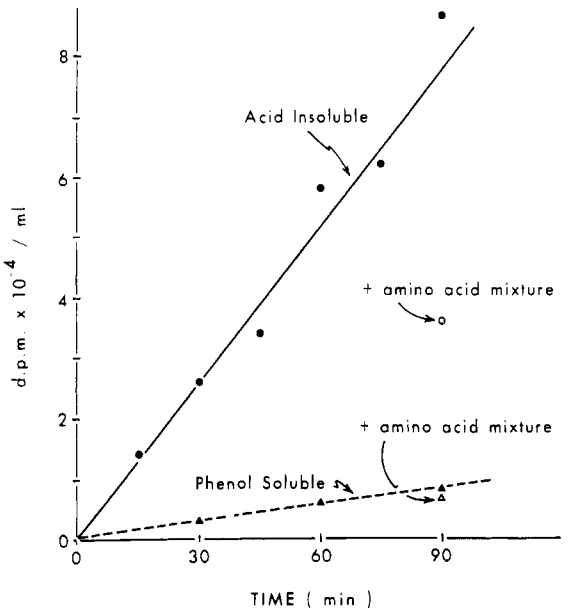

FIGURE 1: Incorporation of *N*-acetylglucosamine-^{14}C into acid-insoluble or phenol-soluble products. The complete amino acid mixture (50 μg/ml) was added at zero time and samples for acid-insoluble products (○) or phenol-soluble products (△) were taken at 90 min.

temperature dependent and could be done at 70, 25, or 4°. After reextracting the combined interface, phenol layer, and residue with glass-distilled water at 4°, the clear phenol layer was dialyzed at room temperature against two to three changes of 0.4% sodium dodecyl sulfate. When the material was clear and free of phenol, it was dialyzed against glass-distilled water, until there was faint turbidity. The turbidity was easily removed by centrifugation. This material was subjected to: acrylamide gel electrophoresis, analytical ultracentrifugation, viscosity measurements, amino acid analysis, immunologic studies, and examination of its biosynthesis *in vivo*.

Studies on Glycoprotein Biosynthesis. *N*-Acetylglucosamine-^{14}C (GlcNAc-^{14}C)[1] was prepared from glucosamine-UL-^{14}C (International Chemical and Nuclear Co.) and acetic anhydride by a modification of the procedure of Roseman and Ludowieg (1954). The GlcNAc-^{14}C was at least 96% pure as determined by paper chromatography in 1-butanol–acetic acid–water (66:10:27). The specific activity was 11.7 mCi/mmole.

Previous workers (Blevins and Dobrogosz, 1967) had shown that GlcNAc but not glucosamine could be incorporated into cell polymers. The GlcNAc-^{14}C was added to log-phase cells (88.4 mμCi/ml of culture). The cells were allowed to grow for various periods of time at 37°. Total incorporation of isotope into acid-insoluble products was measured by collecting aliquots of cell suspensions on a Millipore filter (HA, 0.45 μ), washing three times each with cold 5% trichloroacetic acid (10 ml), and counting the dried filter in a Packard liquid scintillation spectrometer. The scintillation solution used was composed of 150 ml of toluene, 1.5 g of PPO, and 75 mg of POPOP. The remaining cells were extracted with phenol and dialyzed as described above, in order to determine GlcNAc-^{14}C incorporation into the phenol-soluble protein. Aliquots (0.1 ml) of the aqueous sample were measured by liquid scintillation using a solution composed of 208 g of naphthalene, 13 g of PPO, 130 mg of POPOP, 1 l. of xylene, 1 l. of dioxane, and 600 ml of ethanol.

Electrophoretic Techniques. Acrylamide gel electrophoresis was carried out under three sets of conditions. The first condition was in Tris-glycine buffer (pH 8.6) with 7.5% acrylamide according to the method of Ornstein (1964). The second condition involved the same buffer system, but the gels were prepared in 8 M urea (Weinbaum and Markman, 1966). The third electrophoretic condition was in acetic acid and urea (Rottem and Razin, 1967). High-voltage paper electrophoresis was carried out in a Savant Model FP-22A flat-plate instrument using Whatman No. 3MM paper and the formic acid–acetic acid buffer of Lindahl *et al.* (1965). Electrophoresis was at 3000 V for 60 min.

Immunological Techniques. Rabbit antiserum was prepared against isolated envelopes. Agar double diffusion was used to indicate a precipitin reaction and as an index of cross-reactivity of antigens.

Analytical Methods. Protein was determined by the method of Lowry *et al.* (1951). For total carbohydrate the anthrone procedure (Spiro, 1966) was used, and for measurement of reducing sugars after hydrolysis with 1 N HCl for 5 hr on a steam bath, the method of Park and Johnson (1949) was used. Phospholipid phosphorus was analyzed by the method of Shibuya *et al.* (1967).

Results

Incorporation of N-Acetylglucosamine-^{14}C. When GlcNAc-^{14}C was administered to a log-phase culture of *E. coli* B, it was readily incorporated into the cells. The distribution of the radioactivity in acid-insoluble or phenol-soluble products is seen in Figure 1. It is well known that GlcNAc is a major component of two surface macromolecules in *E. coli*, the peptidoglycan (Weidel and Pelzer, 1964) and the lipid portion of the lipopolysaccharide (Burton and Carter, 1964). Incorporation of GlcNAc into these polymers would be included in the acid-insoluble products. As can be seen in Figure 1, addition of a complete mixture of amino acids to the synthetic medium (50 μg/ml final concentration) just prior to addition of GlcNAc-^{14}C reduced the specific activity in the acid-insoluble products by 60%. However, when the phenol-soluble products were examined, as can be seen in Figure 1, the addition of exogenous amino acids had little effect on the radioactivity in this fraction. The difference in the apparent depression of GlcNAc incorporation into the acid-insoluble fraction by amino acids as compared with the phenol-soluble fraction is not well understood. This phenol-soluble fraction accounts for almost 30% of the GlcNAc incorporated into specific polymers (Weinbaum and Okuda, 1968). In order to determine the localization of

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: GlcNAc-^{14}C, *N*-acetylglucosamine-^{14}C; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; SDS, sodium dodecyl sulfate.

TABLE I: Yield of Phenol-Soluble Protein from Various Cell Fractions.

Fraction	Phenol-Soluble Protein / Total Protein in Fraction
Whole cells	0.08–0.10
Envelopes	0.35–0.45
10,000*g* supernatant	0.04–0.08
100,000*g* supernatant	0.01–0.02
Partially purified membranes	0.50–0.60

this material the bacterial cell was fractionated and fractions were extracted as described below.

Cell Fractionation and Localization of the Phenol-Soluble Component. Whole cells of *E. coli* B were fractionated as described in Experimental Procedure, and each fraction was extracted by the phenol method. The results are described in Table I. The material extracted into the phenol phase appears to be a primary component of the cell membrane or cell envelope of the bacterium. If the cells are labeled with either leucine-2-^{14}C or GlcNAc-^{14}C, the percentage of phenol-soluble counts relative to the total incorporation appears to be coincidentally the same with both precursors. Therefore, the material may be either a glycoprotein or a protein closely associated by noncovalent linkage to a carbohydrate. In order to more clearly establish the character of the phenol-soluble material, a criterion of purity as well as a chemical and physical analysis of the material was compiled.

Homogeneity and Chemical and Physical Analyses of the Phenol-Soluble Material. The purity and homogeneity of the phenol-soluble component were determined by three procedures. The first procedure was acrylamide gel electrophoresis in at least three different systems. These results are seen in Figure 2. Gel A, which was run in Tris-glycine buffer (pH 8.6), shows a single, sharply staining, fast-moving band. This pattern varies somewhat and since it appears to be dependent upon the amount of detergent remaining after dialysis, the variation may be due to partial aggregation occurring with loss of detergent. However, at this pH there is always the major fast band. If the electrophoresis is run at pH 8.6 in 8 M urea, as in gel B of Figure 2, the single component in the absence of urea disaggregates into at least four components in the presence of 8 M urea. The disaggregation is not complete since some of the fast band is still present. Finally, if the electrophoretic procedure involves acetic acid plus 5 M urea, as in gel C of Figure 2, the sharp band of gel A gives rise to two very major and at least three to four minor bands. These data suggest that at neutral pH the protein is in an aggregate form, which partially disaggregates at neutral pH in the presence of urea and which more completely disaggregates at acid pH in the presence of urea.

The second procedure for testing the physical properties of the phenol-soluble material was analytical ultracentrifugation. A single peak was obtained as can be seen in Figure 3. If the material was centrifuged in 0.1% SDS the uncorrected s_{20} is 1.5 S, while in 0.01 M Tris buffer (pH 7.0) the s_{20} is 2.9 S. Viscosity measurement at 20° gave a specific viscosity, η, of 24, showing a highly asymmetric particle. This suggestion has been supported by evidence gathered by negative staining of the material and examination by electron microscopy.[2] The data obtained from ultracentrifugation, therefore, have been used only to show that the material appears to be homogeneous under two sets of conditions.

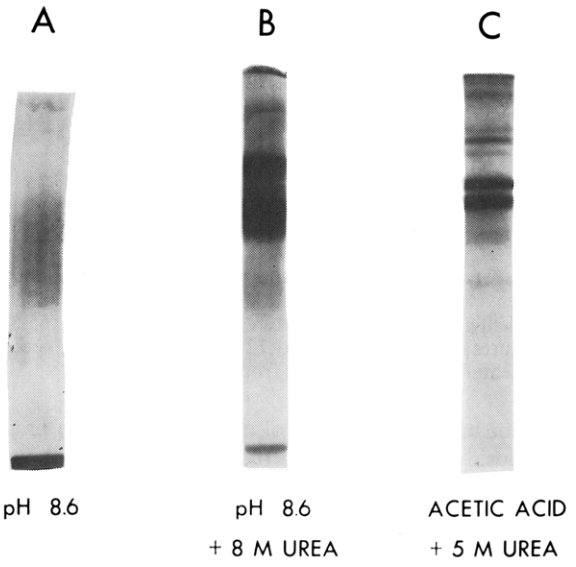

FIGURE 2: Acrylamide gel electrophoresis of phenol-soluble components from envelopes of *E. coli* B.

The third method of establishing purity was Sephadex gel filtration, using Sephadex G-100. Using 0.1% SDS in phosphate buffer (pH 7.0), the material migrated as a single peak close to the void volume. For a nonasymmetric protein this would establish a molecular weight of greater than 50,000. However, because of the highly asymmetric character of this material the lack of agreement between gel filtration and sedimentation data will require additional investigation. We are presently attempting other approaches to elucidate the physical organization of the material.

The phenol-soluble component of the cell envelope, after dialysis against 0.4% SDS and glass-distilled water, was analyzed for protein, carbohydrate, phospholipid, nucleic acid, and mucopeptide. The results are given in Table II. There appears to be 4% carbohydrate and 50% phospholipid relative to protein.

Amino acid analysis of isolated envelopes and the protein component in the phenol are compared in Table III. The envelope amino acid composition is in good agreement with the data presented by Salton (1964). The membrane protein is enriched primarily in aspartic acid and tyrosine. It is of interest to note that there is no

[2] D. A. Fischman and G. Weinbaum, manuscript in preparation.

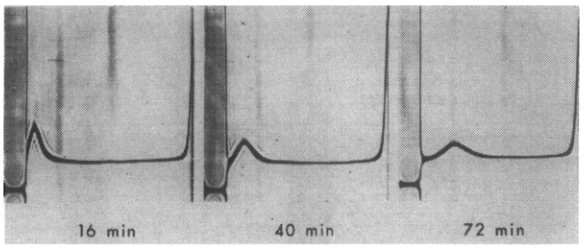

FIGURE 3: Sedimentation of the phenol-soluble component from envelopes of *E. coli* B. Sample was 4.6 mg/ml in 0.05 M Tris buffer (pH 7.5). Schlieren phase-plate angle 65.5°. Exposures were at 16, 40, and 72 min after reaching speed. Sedimentation in all pictures is from left to right.

cysteine and a low content of methionine in both preparations. Spectral analysis of the protein shows an $E_{278/260}$ of 1.56. The $E_{278}^{1\ mg/ml}$ is 1.10.

All of the components described in Table II (*i.e.*, protein, carbohydrate, and phospholipid) are quantitatively precipitated in the presence of 0.02 M Mg^{2+} or Ca^{2+} and aggregate appreciably in the presence of other di- and trivalent cations. This Mg^{2+}-induced aggregation is similar to that observed in *Mycoplasma* (Terry *et al.*, 1967) and in *M. lysodeikticus* (Grula *et al.*, 1967) and is inhibited by pretreatment of the protein with trypsin or more effectively by Pronase. The aggregation is also prevented by extraction with 1-butanol, but addition of the dialyzed butanol fraction to the aqueous portion reestablishes divalent cation induced aggregation. Neither the protein alone nor the phospholipid is precipitated by Mg^{2+}. The aggregate, once formed, can be resolubilized by 0.02 M EDTA. Material labeled with either GlcNAc-^{14}C or leucine-2-^{14}C was quantitatively aggregated by Mg^{2+}.

Mild acid hydrolysis of the phenol-soluble material (1 N HCl, 5 hr at 100°) releases glucosamine and at least one other $AgNO_3$-reducing substance with an R_{GlcNH_2} of 3.14. If the phospholipid is removed by chloroform–methanol or 1-butanol, mild acid hydrolysis of the aqueous phase releases the same reducing substances. If the material labeled with GlcNAc-^{14}C is hydrolyzed both the glucosamine and the unknown reducing substance are radioactive. The mild hydrolysis conditions release about 60% of the radioactivity incorporated from GlcNAc-^{14}C, but less than 10% of the radioactivity incorporated from leucine-2-^{14}C, suggesting that there may be long oligosaccharide portions attached to a small portion of the protein, as well as short carbohydrate side chains on the remainder of the protein.

TABLE II: Chemical Analysis of the Phenol-Soluble Component of *E. coli* B Envelopes.[a]

Component	mg/ml
Protein	4.4
Carbohydrate	0.18
Phospholipid	2.1
DNA + RNA[b]	Negative
Mucopeptide[c]	Negative

[a] Starting material was bacterial envelopes (18 mg/ml dry weight) containing 10.8 mg/ml of protein. [b] DNA was analyzed by the method of Burton (1956), and RNA was analyzed by the procedure of Hurlbert *et al.* (1954). [c] Mucopeptide was determined by the presence of diaminopimelate-2-^{14}C in this fraction after labeling the cells for 2 hr.

TABLE III: Amino Acid Composition of *E. coli* B Envelopes and Phenol-Soluble Protein from Envelopes.

Amino Acid	Envelopes (g/100 g of envelopes)	Phenol-Soluble Protein (g/100 g of protein)
Aspartic acid	7.8	10.5
Threonine	2.5	2.9
Serine	2.0	2.5
Glutamic acid	6.6	6.5
Proline	1.7	1.5
Glycine	3.1	3.9
Alanine	4.3	4.0
Half-cystine	0	0
Valine	3.7	3.7
Methionine	1.4	1.4
Isoleucine	2.7	2.6
Leucine	4.9	4.6
Tyrosine	3.5	5.2
Phenylalanine	3.1	3.1
Lysine	3.5	4.3
Histidine	1.1	0.7
Arginine	4.0	3.7

Characterization of the Phenol-Soluble Component as Envelope-Specific Glycoprotein. Experimentation was needed to establish with certainty that the envelope-specific phenol-soluble material contains carbohydrate as part of its molecular structure. Extraction of the complex with $CHCl_3$–CH_3OH (2:1) removed the phospholipid. Hydrolysis of the protein residue with 1 N HCl for 5 hr released glucosamine as determined by paper chromatography. Still, it was necessary to show covalent linkage. Some methods for attaining this objective were suggested by Murachi *et al.* (1967). The phenol-soluble material was labeled *in vivo* by growing *E. coli* B in the presence of leucine-2-^{14}C or GlcNAc-^{14}C as described in Experimental Procedure. The radioactive purified material was subjected to various treatments as described in Table IV. The data show that in the control the carbohydrate portion is quantitatively precipitated with the protein in the presence of cold 5% trichloroacetic acid. The precipitation is also quantitative in the presence of 0.02 M divalent cation. Treatment with Pronase releases 95% of the protein and 65% of the carbohydrate into the acid-soluble fraction. These data suggest that there are a few different carbohydrate portions associated with the protein. One is easily released by pronase and may be short oligo- or monosac-

charides linked to the protein, while the other linkage involves 35% of the carbohydrate associated with 5% of the protein. This carbohydrate is modified by mild H_2SO_4 treatment and then becomes easily solubilized by Pronase. A combination of Pronase and papain is no more efficient than Pronase alone. Multiple additions of Pronase for 6 days are no more effective than a single overnight incubation.

The glycopeptides formed by the action of Pronase were isolated on Sephadex G-25 to remove the free amino acids, concentrated, and separated by high-voltage electrophoresis. Three radioactive, ninhydrin-positive spots were observed. One major glycopeptide migrated at a speed intermediate to glucosamine and aspartic acid. Mild acid hydrolysis of the glycopeptide mixture yielded radioactive glucosamine as determined by paper chromatography. Dinitrophenylation of the Pronase digest was carried out by the method of Grebner *et al.* (1966) and the derivatives were extracted with ether to extract most DNP-amino acids and then ethyl acetate to extract DNP-glycopeptides and some DNP-amino acids (Greenstein and Winitz, 1961). The extracts were chromatographed on thin-layer chromatography (ITLC-SA, Gelman Instr. Co.) using 1-butanol–5% ammonia (1:1, v/v, upper phase) as the solvent system. Three radioactive DNP-glycopeptide derivatives were observed in the ethyl acetate extract. The extract was evaporated to dryness, hydrolyzed in 2 N HCl for 3 hr at 100°, cooled, and extracted with ethyl acetate, and the HCl fraction was neutralized. The ethyl acetate extract of the hydrolyzed DNP-glycopeptides now showed DNP-Asp and DNP-Tyr by thin-layer chromatography. The neutralized aqueous fraction yielded radioactive glucosamine.

Finally, glycoprotein labeled with GlcNAc-^{14}C was analyzed by gel electrophoresis. Unstained gels were sliced and counted by liquid scintillation spectrometry. Using Tris-glycine (pH 8.6) (Figure 2, gel A) only one radioactive peak was observed corresponding to the fast-moving protein stained on a duplicate gel. When run in acidic conditions (Figure 2, gel C) four of the proteins contained radioactivity. These data suggest that the fast protein in gel A and its subunits in gel C are glycoproteins and tend to reduce the possibilities that (1) there is an oligosaccharide binding to the protein which is noncovalently held and is independent of pH and (2) there is nonspecific polysaccharide contamination. To eliminate these possibilities the biosynthesis of the glycoprotein was examined *in vivo*.

Biosynthesis of the Glycoprotein in Vivo. Incorporation of leucine-2-^{14}C or GlcNAc-^{14}C into glycoprotein was measured under normal growth conditions and in the presence of various inhibitors of protein synthesis (Table V). The data show the necessity of maintaining protein synthesis in order to have glycoprotein synthesis, regardless of which precursor is used. This tends to eliminate the argument of nonspecific polysaccharide contamination. Examination of GlcNAc-^{14}C incorporation into lipopolysaccharide in the presence of chloramphenicol (20 μg/ml) shows it to be essentially unaffected. This suggests that the synthesis of the oligosaccharide portion of the glycoprotein is dependent on protein synthesis.

TABLE IV: Treatment of *N*-Acetylglucosamine-^{14}C Glycoprotein with Various Agents.

Treatment	% Solubilized[a]
None	0
NaOH, 0.5 N at 22°, 30 min	6
HCl, 1.0 N at 100°, 5 hr	61
H_2SO_4, 0.05 N at 80°, 1 hr	10
Pronase,[b] 37°	
Overnight	70
Overnight with leucine-2-^{14}C glycoprotein	95
2 days	66
6 days with fresh addition[c]	66
6 days after H_2SO_4 treatment	90
6 days, then papain[d]	65
Mg^{2+}, 0.02 M, 30 min	0

[a] After treatment the per cent solubilized was determined as that amount of radioactivity remaining soluble in cold 5% trichloroacetic acid; in the Mg^{2+} addition no trichloroacetic acid was necessary. [b] Pronase (1 mg/ml) in phosphate buffer (pH 7.5) was added at a final concentration of 100 μg/ml; 3 drops of toluene was added. [c] Ten additions each of 100 μg of Pronase were made during the 6 days. [d] Papain (100-μg/ml final concentration) was added with 5 mM cysteine and 1 mM EDTA.

Species Specificity of the Phenol-Soluble Material. A number of different cell types have been extracted with phenol and the phenol-soluble material subjected to acrylamide gel electrophoresis at pH 8.6. All the cells examined gave the fast-moving band as the major if not the only component. Upon electrophoresis in acetic acid plus 5 M urea the patterns appear to be species specific. Different acidic patterns were observed for phenol extracts of *E. coli* B, nutritionally induced filamentous *E. coli* B, a thymine-requiring strain of *E. coli*, *M. lysodeikticus*, *Streptococcus pyogenes*, *Mycoplasma* sp., and embryonic rat cells. We also found that 40–50% of beef heart mitochondrial protein is phenol soluble with a rapidly migrating protein band in electrophoresis at pH 8.6 and a characteristic pattern when electrophoresed in acid conditions.

To determine if there was any similarity between the various phenol-soluble protein fractions use was made of antisera prepared against isolated envelopes of *E. coli* B. Using double diffusion in agar, a broad precipitin band was observed between the antisera and the *E. coli* B glycoprotein (Figure 4). The antisera did not react with the high-speed cell supernatant. An extremely interesting finding was that the antisera also reacted with the phenol-soluble fraction from the beef heart mitochondria as well as mitochondrial structural protein isolated by the method of Criddle *et al.* (1962). If the antibody is adsorbed by treating the antisera with an excess of glycoprotein the reactivity against structural

TABLE V: Incorporation of Leucine-2-^{14}C or *N*-Acetylglucosamine-^{14}C into Total Cell Polymers and Membrane-Specific Glycoprotein.

System (1)	DPM Added ($\times 10^{-6}$) (2)	Incorporation into: Acid Insoluble ($\times 10^{-6}$) (3)	Incorporation into: Phenol Soluble ($\times 10^{-6}$) (4)	$\frac{(4)}{(3)} \times 100$
Leucine-2-^{14}C				
Control	2.10	1.04	0.20	19.2
+CM[a] (2 μg/ml)	3.05	2.70	0.59	21.8
+CM (6 μg/ml)	1.88	1.21	0.23	19.0
+CM (10 μg/ml)	2.29	0.73	0.13	17.8
+CM (20 μg/ml)	1.79	0.066	0.014	21.2
+PEA[b] (2.5 mg/ml)	2.54	0.48	0.089	18.6
	($\times 10^{-5}$)	($\times 10^{-4}$)	($\times 10^{-4}$)	
GlcNAc-^{14}C				
Control	1.54	5.5	1.43	26.0
+CM (20 μg/ml)	1.53	0.35	0.087	24.8

[a] CM, chloramphenicol prepared as a stock solution of 1 mg/ml in synthetic medium. [b] PEA, phenethyl alcohol prepared in a stock solution of 100 mg/ml in synthetic medium.

protein is lost. The reverse is also true. The intersection of the precipitin lines against glycoprotein and structural protein appears to suggest cross-reactivity. This aspect is being pursued further by use of immunoelectrophoresis and further purification of glycoprotein as well as structural protein. The presence of lipid in the complex could conceivably account for the broad lines of precipitate. This is being examined by preparing lipid-free glycoprotein and examining the immunologic properties.

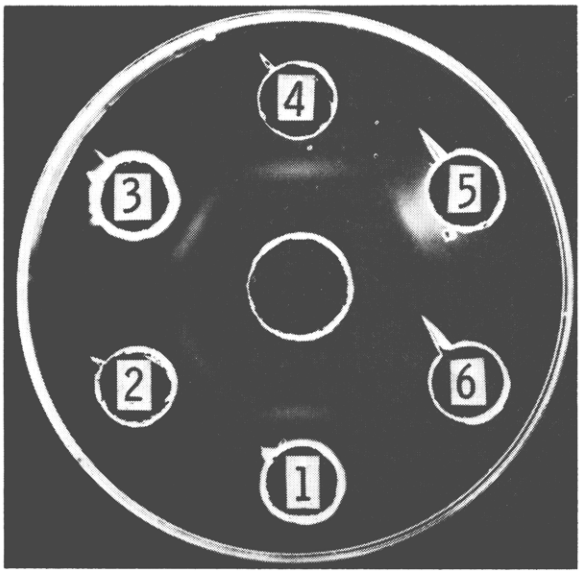

FIGURE 4: Agar double diffusion of various phenol-soluble antigens using antisera against envelopes of *E. coli* B. Center well contains antisera. Well 1: glycoprotein from 10,000*g* supernatant of *E. coli* B; well 2: phenol-soluble beef heart mitochondrial protein; well 3: glycoprotein from envelopes of *E. coli* B; well 4: glycoprotein from envelopes of nutritionally induced filamentous *E. coli* B; well 5: beef heart mitochondrial structural protein; well 6: 100,000*g* supernatant of *E. coli* B.

Discussion

The use of organic solvents to preferentially extract glycoproteins of erythrocyte membranes has been recently demonstrated (Blumenfeld, 1968). Phenol extraction of trypsinized cell envelopes of *Pseudomonas aeruginosa* by Clarke *et al.* (1967) showed that approximately 19% of the envelope protein was in the phenol layer. This protein fraction contained glucosamine and showed a pattern in the analytical ultracentrifuge similar to our envelope-specific glycoprotein from *E. coli* B.

The exact linkage of glucosamine to Asp or Tyr has not been determined yet. It is most likely an *N*-glycosidic linkage similar to what has been found in hen egg albumin (Fletcher *et al.*, 1963). The lack of alkali lability in releasing *N*-acetylglucosamine strongly argues against an *O*-glycosidic link. Tyr has not been implicated in any well-studied glycoprotein.

It is quite possible that glycoproteins are as prevalent in bacterial membranes as they are in animal membranes, though their presence has not yet given an indication of their function. The fact that there is no temperature dependence for the glycoprotein to be extracted into phenol suggests that some membrane-specific biological activity may still be present when extraction is performed at 4°, and our preparations are presently being examined for such possibilities. It is possible that the major role of the envelope-specific glycoproteins is primarily a structural matrix upon which other enzymatically active proteins aggregate. However, in addition to

such a passive role, two other possible functions of a glycoprotein can be suggested. The first possibility is that the carbohydrate portion is necessary in order to set discrete areas of hydrophilic character along the membrane to aid in such biological functions as permeability or phage-mediated injection of nucleic acid. The second and more experimentally verifiable possibility is that the *N*-acetylglucosamine residues on the envelope-specific glycoprotein are actually initiation points for peptidoglycan synthesis, and that the long carbohydrate portion of the glycoprotein is the initiation site for lipopolysaccharide synthesis. This would suggest why aspartic acid is always found as a contaminating amino acid when peptidoglycan is isolated from *E. coli*. The nutritionally induced filaments of *E. coli* B (Weinbaum, 1966) are known to have biologically and morphologically altered membranes (Fischman and Weinbaum, 1967), and we have data suggesting that the altered membranes lack much of their glycoprotein (Okuda *et al.*, 1968). Therefore, it appears that we now have a reversible biological system in which to examine the function of envelope specific glycoproteins.

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